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A mutation in the gene involved in sister chromatid separation causes a defect in nuclear mRNA export in fission yeast

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Abstract

Fission yeast *ptr4-1* is one of the mRNA transport mutants that accumulate poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature. We cloned the *ptr4*$^+$ gene and found that it is identical with the *cut1*$^+$ gene essential for chromosome segregation during mitosis. *ptr4*/*cut1* has no defects in nucleocytoplasmic transport of a protein, indicative of a specific blockage of mRNA export by this mutation. A mutant of Cut2p cooperating with Cut1p in sister chromatid separation also showed defective mRNA export at the nonpermissive temperature. Our results suggest a novel linkage between the cell division cycle and nuclear mRNA export in eukaryotic cells.

Keywords: Ptr4p; Cut1p; mRNA export; Chromosome segregation; Fission yeast

In eukaryotic cells, transport of mRNA from the site of transcription (nucleus) to the site of translation (cytoplasm) occurs via a large proteinaceous structure termed a nuclear pore complex (NPC), which is embedded in the nuclear membrane. Translocation of mRNAs through the NPCs is thought to require coordinated interaction between nucleoporins (nuclear pore complex proteins) and shuttling transport receptors (for reviews, see [1–3]).

Most of the genes affecting nuclear mRNA export were identified by genetic screening in yeasts. Screening for mutants that accumulate poly(A)$^+$ RNA in the nucleus at the nonpermissive temperature using in situ hybridization with the oligo(dT) probe [4–7], or screening for genetic interactions with nucleoporins or other transport-related factors [8,9] revealed a wide variety of genes involved in mRNA export. These include genes for nucleoporins and nuclear pore-associated proteins (Nup85p/Rat9p, Nup120p/Rat2p, Nup145p/Rat10p, Nup159/Rat7p, Gle1p, and Gle2p), RNA binding proteins (Mex67p, Yra1p, Hrp1p, and Npl3p/Mtr11p), and DEAD box RNA helicase (Dbp5p/Rat8p and Sub2p) (for reviews see [1–3,10]). Orthologues for many of these mRNA export-related proteins in mammals have been identified, which means that the basic mechanism involved in mRNA export is conserved among species.

Several lines of experiments suggested that pre-mRNA splicing is directly coupled with mRNA export [1–3]. Aly/REF and a splicing factor UAP56, the yeast orthologue of which is Sub2p, are thought to play key roles in that coupling [9,11]. By injecting labeled mRNA into the nuclei of *Xenopus* oocyte, spliced mRNAs were shown to be preferentially exported to the cytoplasm, as based on their specific association with Aly/REF [11,12]. It was later found that the splicing machinery deposits a large protein complex named an exon–exon junction complex (EJC), which contains Aly/REF, 20–24 nucleotides upstream of exon–exon junctions [13]. The EJC is believed to be the complex responsible for enhancing export of spliced mRNAs by providing a binding platform for Aly/REF [14]. On the other hand, the spliceosomal component Sub2p (the DEAD box helicase) involved in an early step of pre-mRNA splicing is

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thought to recruit Yra1p to the mRNA by direct binding [9]. These factors couple the machineries that function in splicing and export of mRNA.

Recent studies revealed that both Yra1p and Sub2p are stoichiometrically associated with the heterotetrameric THO complex, which functions in transcription in *Saccharomyces cerevisiae* [15,16]. Aly and UAP56 also form a complex with a human counterpart of the THO complex [15]. This conserved complex was designated the TREX (transcription/export) complex and thought to link between gene transcription and mRNA export. These findings led to the view that an extensive coupling network among gene expression machines has evolved to coordinate the efficient expression of genes in the eukaryotic cells [17].

To identify novel factors involved in mRNA export from the nucleus, we isolated six mutants (*ptr1* to *ptr6*) that accumulate poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature in fission yeast *Schizosaccharomyces pombe* [7,18]. We here report molecular cloning and characterization of the *ptr4*$^+$ gene. In addition to the accumulation of poly(A)$^+$ RNA in the nuclei, *ptr4-1* showed aberrant cell division, that is, cytokinesis occurred without prior nuclear division, at the nonpermissive temperature. The *ptr4-1* mutation was found to reside within the *cut1*$^+$ gene involved in sister chromatid separation. To our knowledge, this is the first report suggesting functional linkage between cell division cycle and mRNA export.

Materials and methods

Strains, media, and culture. The *S. pombe* strains used in this study are as follows: T605 (*h*$^-$, *ptr4-1*, *leu1-32*), 972 (*h*$^-$), 975 (*h*$^+$), *cut1* (*h*$^-$, *leu1-32*, *cut1-645*), and *cut2* (*h*$^-$, *leu1-32*, *cut2-364*). The complete media YE and minimum medium MM were used for standard cultures of *S. pombe* [19]. The general genetic method used for *S. pombe* was as described [19].

Fluorescent in situ hybridization. To examine the cellular distribution of poly(A)$^+$ RNA in yeast, fluorescent in situ hybridization was done essentially as described [20]. Briefly, cells shifted to the nonpermissive temperature of 37 °C for the indicated times were fixed with 4% paraformaldehyde and subjected to in situ hybridization with the biotin-labeled oligo(dT)$_{50}$ probe, followed by treatment with FITC conjugated avidin. The samples were finally stained with DAPI and observed under an Olympus AX70 fluorescence microscope equipped with a Photometrics Quantix cooled CCD camera.

To demonstrate that the nuclear signals detected by in situ hybridization with the oligo(dT)$_{50}$ probe reflect the accumulation of poly(A)$^+$ RNA, we performed the following control experiments (data not shown). Addition of a 167-fold molar excess of unlabeled oligo(dT)$_{50}$ oligonucleotide to the hybridization buffer abolished the nuclear signals observed in *ptr4*. In addition, when we used the biotin-labeled oligo(dA)$_{30}$ as a probe, no signals were detected after hybridization. Furthermore, pretreatment of cells with RNase A and RNase T2, a ribonuclease that cleaves preferentially at adenylic bonds, before hybridization abolished completely the hybridizing signals. From these results, we concluded that in situ hybridization with the oligo(dT)$_{50}$ probe detected poly(A)$^+$ RNA in *S. pombe* cells.

Analysis of nuclear protein import and export. To determine if *ptr4-1* has a defect in nucleocytoplasmic transport of proteins, pR1FPA1 expressing a transcription factor Pap1p tagged with GFP [21] was introduced into *ptr4-1*. After culturing overnight at 26 °C in MM medium with thiamine, the cells were washed in sterilized dH_2O, transferred to fresh MM medium without thiamine, and cultured for a further 16 h. The cells were then shifted to 37 °C for 4 h and treated with leptomycin B at a concentration of 200 ng/ml for 30 min. After that, localization of Pap1p–GFP was examined using the OLYMPUS AX70 fluorescence microscope equipped with the cooled CCD camera.

Results

Cytological phenotypes of ptr4-1

The *ptr4-1* mutant accumulates poly(A)$^+$ RNA in the nuclei at the nonpermissive temperature. We isolated it by the screening of a *S. pombe ts*$^-$ mutant bank with in situ hybridization using the oligo(dT) probe [7]. In addition to the nuclear accumulation of poly(A)$^+$ RNA, *ptr4-1* exhibited aberrant cell division at the nonpermissive temperature, as shown in Fig. 1. In this mutant, septation and cytokinesis proceeded without prior nuclear division at the nonpermissive temperature, resulting in cleavage of the undivided nucleus. In some cases, anucleate cells were also generated by nuclear displacement and subsequent cytokinesis (Fig. 1B). These phenotypes are similar to those of the cell untimely torn (*cut*) mutants, in which septation and cytokinesis occur in the absence of nuclear division (for reviews, see [22,23]). The frequency of cells displaying the *cut*-like phenotype increased after 60 min incubation at the nonpermissive temperature and reached a maximal frequency of approximately 55% 4 h after the temperature shift.

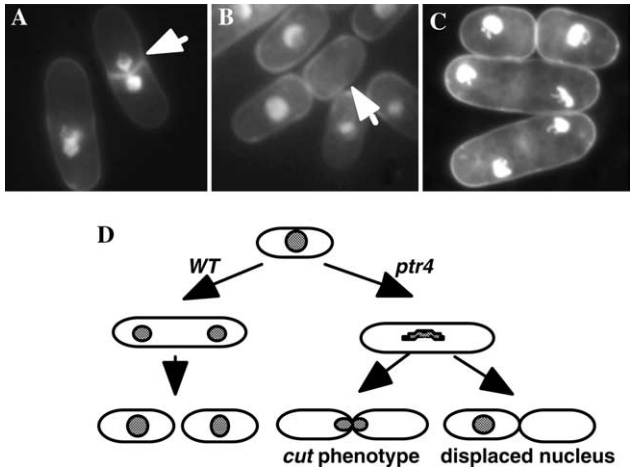

Fig. 1. Cytological phenotype of *ptr4-1*. The *ptr4-1* (A and B) or wild type 972 cells (C) cultured at 26 °C were incubated at 37 °C for 4 h and stained by DAPI. The arrows in (A) and (B) indicate a cell with the *cut* phenotype and an anucleate cell, respectively. (D) A schematic representation of cells with the *cut* phenotype or a displaced nucleus observed in *ptr4-1*.

The anucleate cells were evident at a frequency of about 15% 4 h after shifting to the nonpermissive temperature in *ptr4-1*, as in the case of the *cut2-236* mutant [24].

Cells with the cut phenotype accumulate poly(A)$^+$ RNA in the nuclei

Fig. 2A shows kinetics of nuclear accumulation of poly(A)$^+$ RNA after shifting to the nonpermissive temperature in *ptr4-1*. At 26 °C, poly(A)$^+$ RNA is distributed throughout these cells as in the wild type cells (Fig. 2A-a, time 0′). However, 60 min after shifting to the nonpermissive temperature (37 °C), nuclear accumulation of poly(A)$^+$ RNA was observed. Most of the nuclei accumulating poly(A)$^+$ RNA were located at ends of the cell bodies (Fig. 1A-e, arrowheads). These cells were likely derived from cells with the *cut* phenotype, as treatment of the *cut* cells with the cell wall lysing enzyme during in situ hybridization makes the *cut* cells separate.

The percentage of cells with nuclear accumulation of poly(A)$^+$ RNA increased after a 60 min shift to the nonpermissive temperature in parallel with appearance of the *cut* cells and reached a maximum of approximately 35% 180 min after the shift. Then, intensities of the nuclear signals became weaker, probably due to degradation of the accumulated poly(A)$^+$ RNA.

Two hours after shifting to the nonpermissive temperature, some *ptr4-1* cells with nuclei at the normal position (the center of the cell bodies) showed a strong nuclear accumulation of poly(A)$^+$ RNA (Fig. 2A-i, arrowheads). The percentage of those cells rose in proportion as the anucleate cells increased in number (Fig. 2A-i, arrow) and reached approximately 10% at time of the 4 h shift. The nuclei of these cells strongly stained with DAPI and were larger than the nuclei in normal cells, indicating that they were displaced polyploid nuclei. Thus, not only the *cut* cells, but also the cells with a displaced nucleus seem to be defective in export of poly(A)$^+$ RNA. As far as we analyzed, the nuclear accumulation of poly(A)$^+$ RNA was not evident in dividing anaphase cells (Fig. 2B), suggesting that the nuclear accumulation of poly(A)$^+$ RNA commenced after aberrant cytokinesis had occurred.

Cloning and characterization of the ptr4$^+$ gene

To clone the gene responsible for the *ptr4* mutation, we transformed *ptr4-1* with the *S. pombe* genomic library constructed in a pDB248 vector and incubated the transformants at the nonpermissive temperature of 36 °C. Several transformants which grew at the nonpermissive temperature were isolated. Restriction mapping analysis of the plasmids isolated from the transformants revealed that those clones contained identical genomic fragments. The plasmid also rescued the defects of mRNA export in *ptr4-1* (data not shown).

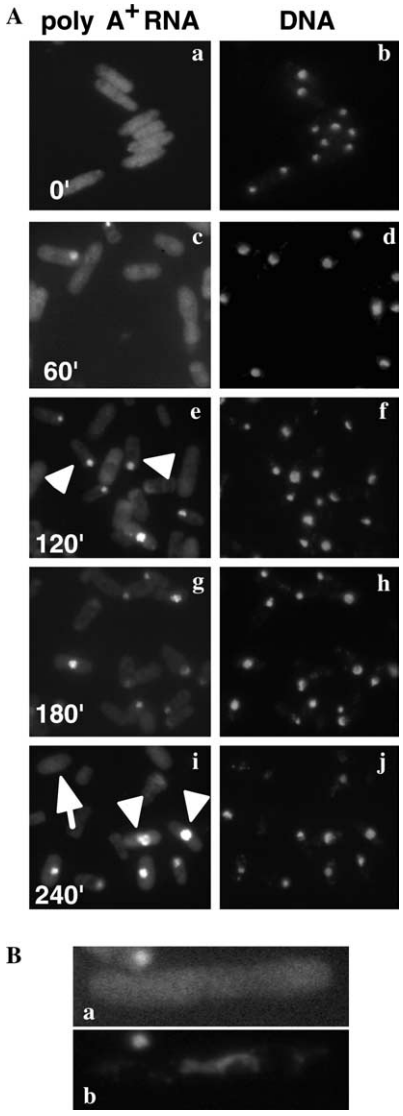

Fig. 2. (A) A kinetics of nuclear accumulation of poly(A)$^+$ RNA after shifting to the nonpermissive temperature in *ptr4-1*. The *ptr4-1* cells cultured at 26 °C were shifted to 37 °C and an aliquot of cells was removed for in situ hybridization with the oligo(dT) probe at 0 min (a), 60 min (c), 120 min (e), 180 min (g), and 240 min (i). b, d, f, h, and j show the DAPI staining of the cells in the corresponding fields. Arrowheads in (e) and (i) indicate cells derived from *cut* phenotype cells and cells with a displaced polyploid nucleus, respectively. An arrow in (i) denotes an anucleate cell. Photographs were taken with the same exposure time. (B) Dividing cells in the mitotic phase showed no accumulation of poly(A)$^+$ RNA in the nuclei. Pictures of a representative cell in the mitotic phase, which was shifted to 37 °C for 180 min, are shown. (a) poly(A)$^+$ RNA, (b) DAPI staining.

An ORF responsible for the complementation of the *ptr4* mutation was identified after several steps of subcloning. A restriction map of the genomic fragment containing the ORF essential for complementation is shown in Fig. 3A. Partial nucleotide sequences of the ORF revealed that the identified gene is identical with the *cut1*$^+$ gene essential for coordinated mitosis, especially for proper sister chromatid separation [24].

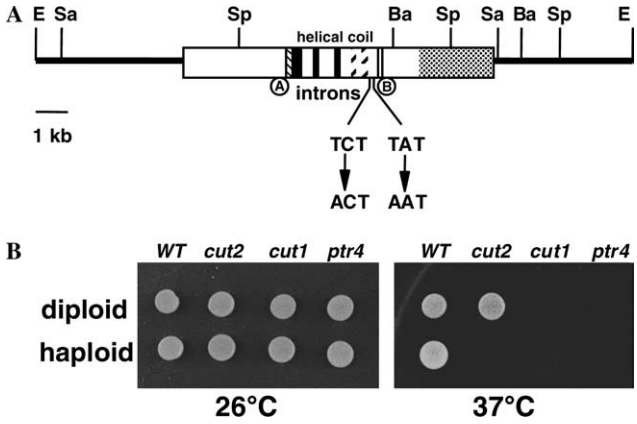

Fig. 3. (A) A restriction map of the cloned *ptr4*$^+$/*cut1*$^+$ gene. The structure of the coding region is shown schematically. Filled boxes denote putative introns. The consensus motifs for ATP binding are indicated by circled (A) and (B). A helical coil region and a carboxy-terminal region homologous to the *S. cerevisiae ESP1* are indicated by striped and shaded boxes, respectively. Two mutation sites found in *ptr4-1* are shown under the restriction map. Restriction sites are abbreviated as follows: E, *Eco*RI; Sa, *Sal*I; Sp, *Spe*I; Ba, *Bam*HI. (B) *ptr4-1* is allelic with *cut1-645*. The first row represents diploid cells constructed by mating *ptr4-1* with *972* (WT) or each mutant strain indicated above. The second row represents haploid cells indicated above. The cells were spotted on a YE plate and incubated at temperatures shown below.

To confirm if *ptr4* is allelic with *cut1*, complementation analysis was done between *ptr4-1* and *cut1-645* or *cut2-364*. *cut2-364* served as a control. After mating, heterozygous diploids were obtained and tested for viability at 37 °C. The diploid with the *ptr4* and *cut1* mutations was temperature-sensitive for growth, which means that *ptr4* and *cut1* are allelic (Fig. 3B). On the other hand, a diploid containing the *ptr4* and *cut2* grew at the nonpermissive temperature.

To determine the mutation site in the *ptr4-1* gene, we amplified the entire region of the ORF in *ptr4-1* and subjected it to sequence analysis. A comparison between the determined sequence and that of the *cut1*$^+$ gene revealed the presence of two point mutations located around the central region of the gene (Fig. 3A). One is located at a nucleotide position of 3487 which changes T to A (a replacement of amino acid serine to threonine) and the other at a nucleotide position of 3505 which changes T to A (a replacement of tyrosine to asparagine).

ptr4-1 has no apparent defects in protein import and export

To determine if the *ptr4-1* mutant has a defect in nucleocytoplasmic transport of a protein, we observed cellular distribution of the Pap1p–GFP fusion protein in *ptr4-1*. The transcription factor Pap1p is a shuttling protein with the leucine-rich NES, the export of which is mediated by the export receptor Crm1p [25]. In wild type cells, Pap1p–GFP predominantly distributed in the cytoplasm both at 26 and 37 °C. In contrast, after addition of leptomycin B (LMB, an inhibitor for Crm1p), the fusion protein quickly accumulated into the nucleus, suggesting that expressed Pap1p–GFP is shuttling between the nucleus and the cytoplasm in the cells (Figs. 4A and B). We observed similar results in *ptr4-1* which was shifted to the nonpermissive temperature. The *ptr4-1* cells with the *cut* phenotype showed the cytoplasmic distribution of Pap1p–GFP at the nonpermissive temperature of 37 °C (Fig. 4C). After addition of LMB, the *cut* nuclei, as well as nuclei not exhibiting the *cut* phenotype, rapidly accumulated Pap1p–GFP, suggesting that *ptr4-1* has no defects in protein import at the nonpermissive temperature (Fig. 4D). Also, the cytoplasmic distribution of Pap1p–GFP in the absence of LMB at 37 °C implies that export of the protein is not affected by the *ptr4* mutation.

cut2 also accumulates poly(A)$^+$ *RNA in the nuclei at the nonpermissive temperature*

Cut1p is known to interact with Cut2p to form a large complex that functions in sister chromatid separation [26]. We next asked if a mutation in Cut2p would

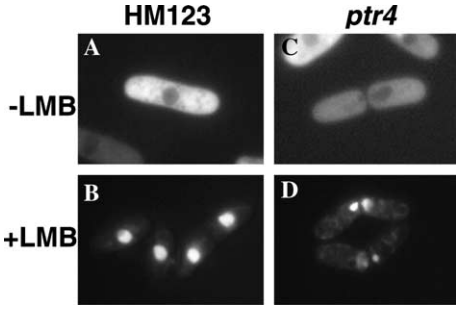

Fig. 4. Analysis of nuclear protein import and export. Wild type (HM123, A and B) and *ptr4-1* (C and D) cells expressing Pap1p–GFP were shifted to the nonpermissive temperature of 37 °C for 4 h. Then, distribution of the GFP-fusion protein was analyzed in the absence (A and C) or in the presence (B and D) of leptomycin B.

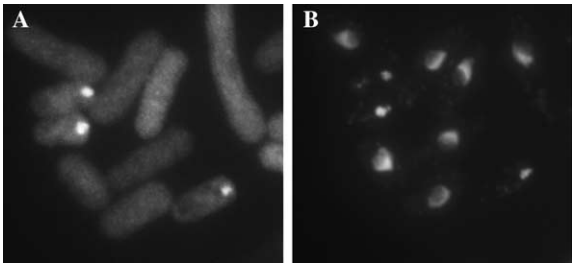

Fig. 5. In situ hybridization of *cut2-364*. The *cut2-364* cells shifted to the nonpermissive temperature for 2 h were subjected to in situ hybridization with the oligo(dT) probe. Cells with the *cut* phenotype accumulated poly(A)$^+$ RNA in the cleaved nuclei. (A) poly(A)$^+$ RNA, (B) DAPI staining.

affect nuclear mRNA export. The *cut2-364* mutant cultured at 26 °C was shifted to the nonpermissive temperature for 2 h and then subjected to in situ hybridization. As a result, we found that the *cut2-364* mutant also accumulates poly(A)$^+$ RNA in the nuclei of cells expressing the *cut* phenotype at the nonpermissive temperature (Fig. 5).

Discussion

We cloned the *ptr4*$^+$ gene and found that it is identical with the *cut1*$^+$ gene involved in sister chromatid separation. *ptr4-1* showed a typical *cut* phenotype in addition to the nuclear accumulation of poly(A)$^+$ RNA at the nonpermissive temperature (Fig. 1). *cut1* was initially identified to be a mutation that causes cytokinesis without prior nuclear division because of a failure of chromosome separation [27]. The *cut1*$^+$ gene encodes a large nuclear protein of 1827 amino acid residues [24]. Cut1p consists of a putative ATP binding domain, a helical coil region, and a carboxy-terminal region homologous to the *S. cerevisiae* ESP1. Localization of Cut1p was reported to alter during the cell cycle [28]. Cut1p distributes in the cytoplasm during interphase and then moves to mitotic spindle pole bodies and spindle upon entry into prophase during mitosis [28]. Localization of Cut1p to the spindles is known to require its association with Cut2p, which is also essential for chromosome segregation [28]. Interestingly, we found that the *cut2* mutation also causes defective mRNA export at the nonpermissive temperature, indicating that aberrant cell division leads to a block of mRNA export.

In the *ptr4*/*cut1* and *cut2* mutants, only cells expressing the *cut* phenotype or cells with a displaced polyploid nucleus accumulated poly(A)$^+$ RNA at the nonpermissive temperature (Fig. 2). As protein import and export in the *cut* phenotype cells are normal (Fig. 4A), functions of NPCs seem to be preserved even after aberrant cleavage of the nucleus, and blockage in nuclear export in *ptr4-1* is specific for poly(A)$^+$ RNA.

At present, we do not know how Ptr4p/Cut1p and Cut2p are involved in mRNA export pathway in a cell and their involvement in mRNA export is direct or not. However, it is noteworthy that Rae1p, an essential mRNA export factor in *S. pombe*, was recently found to function as a mitotic checkpoint regulator in mice [29]. Haplo-insufficiency of mouse Rae1 resulted in a mitotic checkpoint defect and chromosome missegregation [29]. In addition to the coupling system among mRNA export, RNA processing, and gene transcription [17], our results suggest that a linkage between the cell cycle progression and mRNA export also exists in eukaryotic cells. Molecular mechanisms of such novel coupling network in the cells are subjects of ongoing studies.

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